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High temperature enhances inhibitor production but reduces fallover in tobacco Rubisco

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Abstract. High temperature inhibits photosynthesis by several mechanisms including reduction in Rubisco activity. While the initial reaction velocity of purified, fully carbamylated, inhibitor-free Rubisco increases with temperature *in vitro*, over time, the reaction velocity slowly declines (fallover) because of the enzymatic and non-enzymatic production of inhibitors from the substrate ribulose-1,5-bisphosphate. We tested whether fallover could contribute to the decline in Rubisco activity observed in leaf extracts at high temperature. Production of D-xylulose-1,5-bisphosphate (XuBP), an inhibitor of Rubisco, was greater at 35 and 45°C than at 25°C but fallover was less severe at 35 and 45°C than at 25°C, both in rate and extent under saturating CO_2 and ambient O_2. This apparent dichotomy is consistent with the catalytic site of Rubisco loosening at higher temperatures and releasing inhibitors more easily. The loosening of the catalytic site was supported by the observation that RuBP and XuBP were released from their complexes with uncarbamylated, Mg^{2+}-free Rubisco faster at 35 and 45°C than at 25°C. We conclude that, although XuBP production increased relative to catalytic throughput at higher temperatures, this was more than compensated for by its faster release, resulting in less fallover inhibition at higher temperatures.

Keywords: fallover, heat stress, high temperature, Rubisco.

Introduction

High temperature reduces plant growth, with photosynthetic CO_2 assimilation being one of the most sensitive processes to high temperature (Berry and Björkman 1980). This decline in photosynthesis is strongly correlated with the activity of ribulose-1,5-bisphosphate carboxylase / oxygenase (Rubisco) extracted from heat-stressed leaves (Kobza and Edwards 1987; Law and Crafts-Brandner 1999; Schrader *et al*. 2004). The decline in Rubisco activity is not due to a degradation of Rubisco itself, as Rubisco is stable at temperatures as high as 50°C (Eckardt and Portis 1997; Crafts-Brandner and Salvucci 2000), but is the result of decarbamylation of Rubisco's catalytic site and a possible increase in inhibitors bound to Rubisco's catalytic site (Sharkey *et al*. 2001; Salvucci and Crafts-Brandner 2004).

Rubisco is the central enzyme in photosynthetic carbon fixation, catalysing the carboxylation and oxygenation of D-ribulose-1,5-bisphosphate (RuBP). Rubisco also catalyses several side reactions of RuBP. Although these side reactions constitute a minor fraction of the total end products, several are known to, or have been implicated to, cause a slow decline in Rubisco activity *in vitro*, termed 'fallover' (Andrews and Hatch 1969; Edmondson *et al*. 1990*a*). Fallover is thought to result from competitive inhibition by various sugar phosphates produced by Rubisco (Edmondson *et al*. 1990*b*; Kane *et al*. 1998), which is alleviated by the action of Rubisco activase (Robinson and Portis 1989).

The initial catalytic step for all reactions involving Rubisco is the formation of the enediol from RuBP. In addition to the well-known carboxylation and oxygenation

Abbreviations used: BSA, bovine serum albumin; CA, carbonic anhydrase; CtBP, 2′-carboxytetritol-1,4-bisphosphate; PdBP, D-glycero-2,3-diulose-1,5-bisphosphate; Rubisco, ribulose-1,5-bisphosphate carboxylase / oxygenase; RuBP, D-ribulose-1,5-bisphosphate; XuBP, D-xylulose-1,5-bisphosphate.

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reactions, the enediol can misprotonate to form D-xylulose-1,5-bisphosphate (XuBP) or undergo β-elimination to form deoxypentodiulose. If the enediol undergoes carboxylation, then the aci-acid can form pyruvate instead of PGA. If the enediol undergoes oxygenation, the peroxyketone can eliminate H_2O_2 to form D-glycero-2,3-pentodiulose-1,5-bisphosphate (PdBP), which may rearrange to form 2′-carboxytetritol-1,4-bisphosphate (CtBP; Harpel *et al*. 1995). While deoxypentodiulose and pyruvate have not been shown to cause fallover, XuBP has been shown and PdBP and CtBP have been implicated to cause fallover. XuBP is a stable compound and has been isolated for study, but the study of PdBP and CtBP in isolation has been hampered by their instability (Chen and Hartman 1995). Early work demonstrated the prominent role of XuBP in fallover and its accumulation on Rubisco's catalytic site during extended reaction times (Edmondson *et al*. 1990*d*; Pearce and Andrews 2003). However, reactions suppressing XuBP by adding aldolase (Edmondson *et al*. 1990*a*) or increasing CO_2 and O_2 concentrations (Zhu *et al*. 1998) still demonstrated a loss of Rubisco activity. This suggested that other inhibitors may be involved in fallover. These inhibitors, PdBP and possibly CtBP, were identified because they also accumulated non-enzymatically in stored RuBP solutions causing varying results between Rubisco assays (Kane *et al*. 1998). Kane *et al*. (1998) also suggested that PdBP is a more potent inhibitor of Rubisco because of its tighter binding to the catalytic site. Enzymatically, PdBP and CtBP have only been produced by Rubisco mutants or at elevated temperatures by spinach (*Spinacia oleracea* L.), *Chlamydomonas reinhardtii*, and *Rhodospirillum rubrum* Rubiscos (Kim and Portis 2004), suggesting their importance *in vivo* may be limited to elevated temperatures.

High temperature stimulates Rubisco side reactions at the expense of carboxylation. The oxygenation of RuBP (Ogren 1984), the production of pyruvate (Andrews and Kane 1991), the production of PdBP and CtBP under oxygenating conditions (Kim and Portis 2004), and the production of XuBP (Salvucci and Crafts-Brandner 2004) are all elevated at higher temperature. This increase in side products from RuBP, especially XuBP, PdBP, and CtBP, suggests that fallover might be worse at high temperatures. While fallover has been studied in some detail at 25°C, little information is available at different temperatures. Here, we have investigated the phenomenon of fallover and *in vitro* carbamylation in the presence of RuBP or XuBP at various temperatures in isolated, purified, tobacco Rubisco. We also explored some of the temperature effects on Rubisco inhibitor production.

Materials and methods

Materials

Wild type tobacco (*Nicotiana tabacum* L. cv W38) was grown at 27 / 19°C day / night in an artificially illuminated growth chamber with a 15-h photoperiod. Rubisco was isolated by crystallisation as described by Servaites (1985). Rubisco concentration was estimated by absorbance at 280 nm using a molar extinction coefficient of 0.7 (Kung *et al*. 1980; Pearce and Andrews 2003). RuBP was synthesised as described by Kane *et al*. (1998) and stored under liquid nitrogen. XuBP was synthesised as described by Pearce and Andrews (2003).

Fallover (decline of Rubisco activity during catalysis)

Rubisco activity was measured by the incorporation of $^{14}CO_2$ into acid stable compounds. Throughout all radioactive experiments, the specific activity of $^{14}CO_2$ used to assay Rubisco was the same as that used to activate Rubisco unless noted otherwise. Rubisco was activated at a concentration of 9.5 μg mL^{-1} by incubation at 50°C for 10 min and then cooled to room temperature in 20 mM $NaH^{14}CO_3$ (4400 CPM nmol^{-1} for 15 and 25°C and 600 CPM nmol^{-1} for 35 and 45°C), 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, 0.1 mg mL^{-1} carbonic anhydrase (CA), and 0.1 mg mL^{-1} bovine serum albumin (BSA). To initiate the reaction the activated Rubisco was injected, 0.25 μg mL^{-1} final concentration, into sealed glass vials with final concentrations of 0.6 mM RuBP, 20 mM $NaH^{14}CO_3$ (same specific radioactivity as Rubisco activation above), 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, 0.1 mg mL^{-1} CA, and 0.1 mg mL^{-1} BSA with a final volume of 1 mL. Assays were conducted at 15, 25, 35 and 45°C in temperature-controlled water baths. Aliquots (50 μL) were removed and injected into 25% formic acid to stop the reaction. Samples were dried at 80°C and the radioactivity remaining was counted after the addition of scintillation cocktail (Ultima Gold XR, Perkin Elmer, Shelton, CT).

Data for $^{14}CO_2$ fixation *v.* time were fitted to the following standard fallover equation (Pearce and Andrews 2003),

$$^{14}CO_2 \text{ Fixed} = \frac{V_i - V_f}{k_{obs}}\left(1 - e^{-k_{obs}t}\right) + V_f t, \quad (1)$$

where V_i is the initial reaction velocity, V_f is the final reaction velocity, k_{obs} is the observed first-order rate constant, and t is time. Estimates of V_i, V_f and k_{obs} were used in the following differentiated form of Eqn 1 to yield Rubisco activity *v.* time:

$$V = (V_i - V_f)\, e^{-k_{obs}t} + V_f. \quad (2)$$

Rubisco catalysis over time was also assayed in the presence of RuBP that had been pre-incubated at various temperatures before the initiation of the reaction. RuBP, 3.7 mM final concentration, was incubated at 15, 25, 35 or 45°C in 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, and 1 mM EDTA for one hour before assaying Rubisco carboxylation as described above. Final RuBP concentration was the same as above at 0.6 mM.

Rubisco activation

Rubisco activation was measured similarly to assays measuring Rubisco fallover except that Rubisco was incubated in CO_2 and $MgCl_2$-free buffer at a concentration of 10 μg mL^{-1} and containing either 0.08 mM RuBP or 0.08 mM XuBP at 50°C for 10 min and then cooled to room temperature for a minimum of 10 min in 100 mM EPPS–NaOH pH 8.0, 1 mM EDTA, 0.1 mg mL^{-1} CA, and 0.1 mg mL^{-1} BSA before initiation of the reaction. Pre-incubation RuBP and XuBP concentrations were chosen to be saturating for Rubisco sites, 150 nM, and followed the protocol of Pearce and Andrews (2003). Rubisco activity was initiated by injecting the RuBP- or XuBP-complexed Rubisco, at a final concentration of 10 μg mL^{-1}, into an otherwise complete reaction solution with a final concentration of 0.75 mM RuBP, 20 mM $NaH^{14}CO_3$ (800 CPM nmol^{-1}), 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, 0.1 mg mL^{-1} CA, and 0.1 mg mL^{-1} BSA with a final volume of 0.75 mL. Aliquots (50 μL) were removed at 60-s intervals over a 480-s period and injected into 25% formic acid to stop the reaction. Acid-stable C^{14} radioactivity was measured as before.

Data for $^{14}CO_2$ fixation *v.* time were fitted to the following modified fallover equation where V_i was set equal to 0 (Pearce and Andrews, 2003):

$$^{14}CO_2 \text{ Fixed} = V_f t - V_f \frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs}}. \quad (3)$$

Parameters are the same as in Eqn 1.

XuBP production measured spectrophotometrically

XuBP production rates were determined by measuring total XuBP produced after allowing fully activated Rubisco to consume RuBP for 2, 4, 6, and 8 min at 15, 25, 35 or 45°C under non-carboxylating / non-oxygenating conditions achieved by using N_2-sparged buffer. Rubisco was activated as previously described at a concentration of 60 mg mL^{-1}, in the absence of CA and with only 10 mM $NaHCO_3$ so as to reduce the carryover of CO_2 to the assays. Activated Rubisco, 6.7 mg mL^{-1} final concentration, was added to a final concentration of 3.3 mM RuBP, 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, and 1 mM EDTA with a final volume of 0.45 mL that had been sparged with N_2 and equilibrated at the desired temperature. The headspace of the reaction vial was continually flushed with N_2 throughout the course of the reaction. After 2, 4, 6 and 8 min, 100 µL of the reaction mixture was added to perchloric acid, 1% final concentration, to stop the reaction. The mixture was held on ice for 30 min before neutralisation with K_2CO_3. Precipitated $KClO_4$ was removed by centrifugation and 100 µL of the resulting supernatant was added to a spectrophotometer cuvette containing a final concentration of 0.25 mM NADH, 2.0 mg mL^{-1} aldolase, 0.3 mg mL^{-1} glycerol-P dehydrogenase, 100 EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, and 1 mM EDTA, and the change in absorbance at 340 nm (A_{340}) was monitored. The total XuBP present was calculated from the change in NADH absorbance at A_{340}. Carryover $NaHCO_3$ from activated Rubisco was 1.9 mM and at 15°C would have been completely consumed in ~16 s.

Rubisco activity in the presence of enzymatically produced inhibitors

Rubisco carboxylase activity was measured after exposing the enzyme to the reaction products of an earlier Rubisco-catalysed, RuBP-dependent, reaction (Fig. 1). These earlier Rubisco reactions were conducted under (1) carboxylation / oxygenation-limited, (2) saturated carboxylating, or (3) oxygenating conditions. Rubisco, 40 mg mL^{-1} final concentration, was activated by incubation at 50°C for 10 min and then cooled to room temperature in a final concentration of 15 mM $NaHCO_3$, 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, 0.1 mg mL^{-1} CA, and 0.1 BSA, and was injected, 2.0 mg mL^{-1} final concentration, into a final concentration of 7.7 mM RuBP, 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, and 10 mM borate at a final volume of 200 µL that contained either 20 mM $NaHCO_3$ (0% O_2) or had been sparged with either N_2 or O_2. After incubation at 45°C for 10 min, the reactions were then stopped by the addition of perchloric acid, 1% final concentration, held on ice for 30 min, neutralised with K_2CO_3, and filtered with a Millipore (Billerica, MA) Ultrafree-MC filter unit (M_r 10 000 MWCO). 200 µL of the supernatant was added to fresh, activated Rubisco, 11.1 µg mL^{-1} final concentration, in a final concentration of 15 mM $NaH^{14}CO_3$ (3800 CPM nmol^{-1}), 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, and 1 mM EDTA and incubated at room temperature for 30 min. Rubisco was activated as described above except in the presence of 15 mM $NaH^{14}CO_3$ (3800 CPM nmol^{-1}) and at a concentration of 200 µg mL^{-1}. After incubation, the solutions were brought to 15, 25, 35 or 45°C for 2 min and 100 µL of the supernatant / Rubisco mixture was added to a final concentration of 0.7 mM RuBP, 15 mM $NaH^{14}CO_3$ (same specific radioactivity as incubation above), 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, and 1 mM EDTA to initiate carboxylase activity with a final concentration of 2.5 µg Rubisco mL^{-1} and a final volume of 550 µL. Aliquots (100 µL) of the carboxylase reaction were removed at 120-s intervals over a period of 480-s and injected into 25% formic acid to stop the reaction. Acid-stable C^{14} radioactivity was measured as before. Carryover $NaHCO_3$ from activated Rubisco was 0.75 mM in the pre-incubation N_2 and O_2-sparged reactions. A Rubisco control, lacking Rubisco in the initial pre-incubation reaction, and an RuBP control, lacking RuBP in the initial pre-incubation reaction, were also assayed. Both controls were only analysed using the saturating carboxylating condition, 20 mM $NaHCO_3$ (0% O_2), in the pre-incubation reaction. The pre-incubation N_2 and O_2-sparged controls were not assayed.

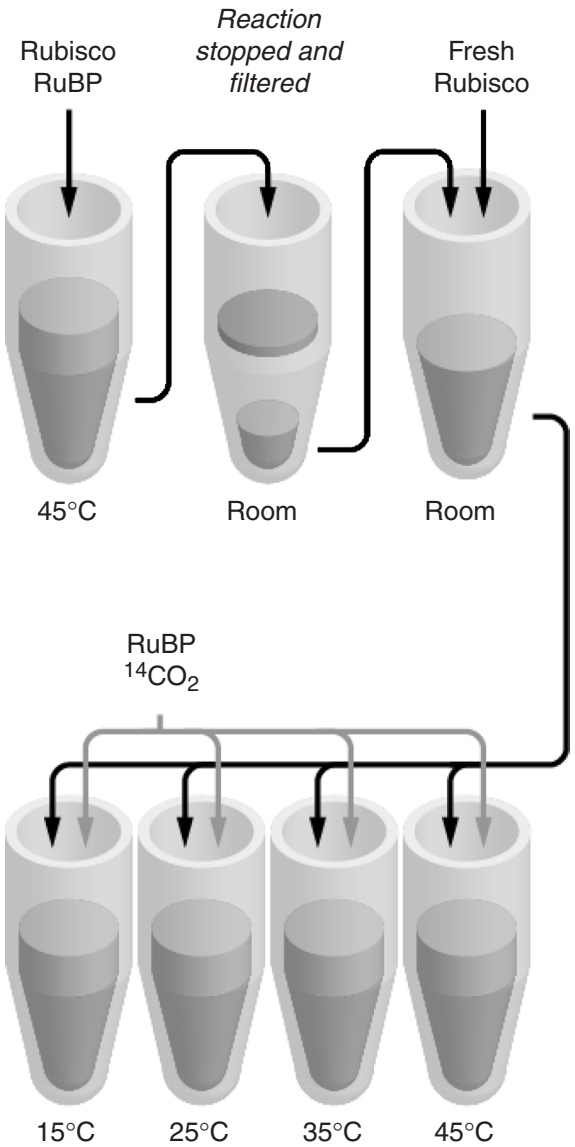

Fig. 1. Illustration of the experimental method used to analyse Rubisco activity in the presence of enzymatically produced inhibitors. Rubisco was initially incubated at 45°C in the presence of RuBP under various atmospheric conditions. The reaction was stopped and filtered to remove Rubisco. Fresh, fully carbamylated Rubisco was added again without RuBP and allowed to equilibrate. Rubisco activity was then measured at 15, 25, 35 and 45°C with saturating RuBP and $^{14}CO_2$. See Materials and methods for details.

Results

Temperature effects on Rubisco fallover

Under saturating CO_2 and ambient O_2, fully carbamylated, Mg^{2+}-bound Rubisco activity declined over time at all four temperatures (15, 25, 35 and 45°C) before reaching a constant activity (Fig. 2). The initial (V_i) and final (V_f) velocity, rate of decline (k_{obs}), and half-time ($t_{1/2}$) of each reaction was derived by fitting the data in Fig. 2*A* to Eqn 1 (see Materials and methods) and are shown in Table 1. The rates in Fig. 2*B* were plotted using Eqn 2 (Materials and methods). Because of the near linearity of data at 45°C, V_f was calculated directly from the last four data points and used to constrain the fitting function to obtain a more accurate estimate of V_i and k_{obs}. For every 10°C increase in temperature, V_i increased ~2-fold, which is consistent with previous reports (Eckardt and Portis 1997; Crafts-Brandner and Salvucci 2000; Salvucci *et al*. 2001). Increasing temperature also decreased the extent ($1–V_f / V_i$) and increased the $t_{1/2}$ of fallover (Fig. 3; Table 1). At 25°C after 60 min, V_f was 46% of V_i with a $t_{1/2}$ of 257 s, consistent with previously reported observations (Edmondson *et al*. 1990*a*), while at 45°C, V_f was 81% of V_i and changed considerably more slowly ($t_{1/2}$ of 1155 s).

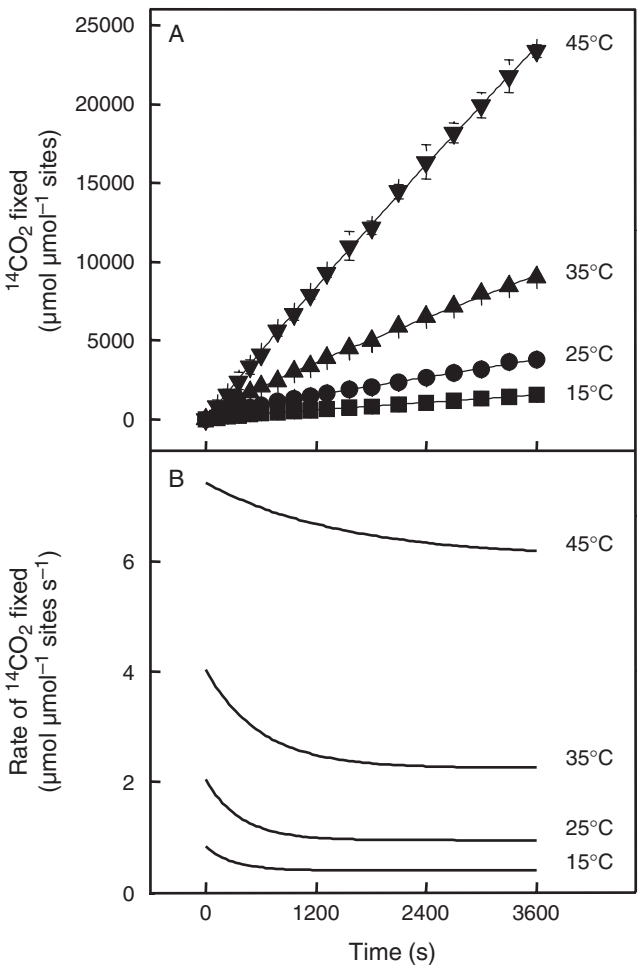

Fig. 2. Slow inhibition of Rubisco activity *in vitro* during catalysis under saturating CO_2 and ambient O_2. Acid-stable Rubisco product accumulation (*A*) at 15 (■), 25 (●), 35 (▲) and 45°C (▼) and the corresponding change in reaction rate over time (*B*). Reaction rates were derived by fitting acid-stable product accumulation data to Eqn 1, estimating V_i, V_f, and k_{obs}, and applying these estimates to plot the reaction rate *v.* time using Eqn 2. Assays were initiated by the additions of preactivated Rubisco (final concentration of 0.25 µg mL^{-1}) to an otherwise complete reaction mixture containing a final concentration of 20 mM $NaH^{14}CO_3$ with air levels of O_2, 0.6 mM RuBP, 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, 0.1 mg mL^{-1} CA, and 0.1 mg mL^{-1} BSA. Rubisco sites were estimated at 1 mg protein = 15 nmol sites. $n = 3$.

To control for the non-enzymatic, spontaneous conversion of RuBP into inhibitors at different temperatures, we also conducted fallover experiments at 25°C with RuBP that had been incubated at 15, 25, 35 and 45°C for 60 min without Rubisco (Table 1). Fallover curves for pre-incubated RuBP were similar to those obtained without pre-incubating RuBP with V_i near 2 except that in all cases V_f was lower when RuBP was pre-incubated. Between the four temperatures, V_f / V_i was not significantly different and the mean V_f / V_i (± s.d.) of all four temperatures was 0.25 ± 0.06.

Temperature effects on Rubisco activation

The activation rate under saturating CO_2 of uncarbamylated, Mg^{2+}-free Rubisco complexed with either RuBP (ER) or XuBP (EX) increased as temperature increased (Fig. 4). Estimates of activation parameters were calculated by fitting the Rubisco product accumulation data *v.* time to Eqn 3 (see Materials and methods), and then plotted using Eqn 2. With both ligands, increasing temperature increased the rate of activation (Table 2). However, the rate of activation was faster for ER than EX at 15, 25 and 35°C, reflecting the inhibition of catalysis by XuBP. Further, ER activated fully to V_i levels determined with fully activated Rubisco except at 15°C. EX complexes, however, did not fully activate even at 45°C, reflecting a temperature-independent inhibition of activity by XuBP, although the degree of inhibition became progressively less as temperature increased. Because activation was only measured over 600 s, the $t_{1/2}$ of EX complexes at 15°C may not be indicative of the true $t_{1/2}$ and better estimates could be obtained by measuring activation over a longer period.

Temperature effects on inhibitor production

Temperature dependent XuBP production was further tested by incubating RuBP in the presence of activated Rubisco under N_2 (Fig. 5). Under these conditions RuBP cannot be carboxylated or oxygenated and is limited to forming either XuBP or deoxypentodiulose-P. XuBP production increased with temperature ~2-fold for every 10°C increase. Rates were determined by the slope of the linear regression through the four time points for each temperature.

Table 1. Kinetic parameters for fully activated Rubisco taken from Figs 1 and 2
Parameters were estimated using Eqn 1 on the mean of three independent replications of each temperature. $n = 3 \pm$ s.d.

Temperature (°C)	V_i (s^{-1})	V_f (s^{-1})	k_{obs} (s^{-1})	V_f / V_i	$t_{1/2}$ (s)
Fully activated Rubisco assayed in 20 mM $NaHCO_3$					
15	0.8 ± 0.04	0.4 ± 0.002	0.0035 ± 0.0007	0.47	198
25	2 ± 0.13	0.9 ± 0.03	0.0027 ± 0.0002	0.46	257
35	4 ± 0.14	2.2 ± 0.02	0.0017 ± 0.0002	0.56	408
45	7.4 ± 0.24	6 ± 0.27	0.0006 ± 0.0001	0.81	1155
Fully activated Rubisco assayed at 25°C in 20 mM $NaHCO_3$ after incubating RuBP for 60 min at the given temperatures					
15	2.4 ± 0.47	0.51 ± 0.02	0.0015 ± 0.0004	0.22	462
25	2 ± 0.02	0.67 ± 0.08	0.0018 ± 0.0004	0.34	385
35	2.3 ± 0.16	0.51 ± 0.14	0.0014 ± 0.0004	0.22	495
45	2 ± 0.18	0.46 ± 0.14	0.001 ± 0.0004	0.23	693

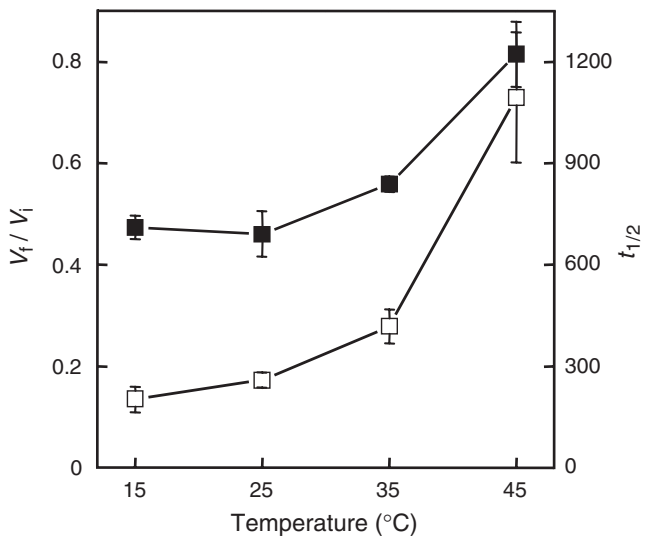

Fig. 3. Temperature effect on the kinetic parameters of the slow inhibition of Rubisco activity *in vitro* during catalysis under saturating CO_2 (V_f / V_i ■, $t_{1/2}$ □). The parameters were taken from those derived in Fig. 2 and Table 1. $n = 3 \pm$ s.d.

Temperature effects on Rubisco activity in the presence of inhibitors formed by Rubisco side reactions

To examine the effects of inhibitors produced by Rubisco at high temperature, RuBP was incubated in the presence of Rubisco at 45°C with either 20 mM $NaHCO_3$ (saturated carboxylating) or reaction mixtures sparged with either N_2 (suppressing carboxylation and oxygenation) or O_2 (predominantly oxygenating). PdBP produced during the course of these reactions was stabilised by the inclusion of 10 mM borate. The reactions were stopped with perchloric acid and the neutralised supernatant was supplied to fresh Rubisco and RuBP, and the carboxylation rates were measured at 15, 25, 35 and 45°C under saturating CO_2 and saturating RuBP (Table 3). For all treatments, Rubisco activity increased with temperature. However, relative to controls, Rubisco activity was lower in all treatments with the most pronounced effect on Rubisco exposed to supernatant from the predominantly oxygenating condition. Presumably, the high O_2 / low CO_2 conditions favoured an increased production of PdBP and CtBP by Rubisco and these are more potent inhibitors of Rubisco. However, the levels of PdBP and CtBP were not quantified in this treatment. The activity of Rubisco incubated with supernatant from oxygenating conditions showed a greater than 2-fold increase from 35 to 45°C.

Discussion

Slow inhibition during catalysis is less at high temperature under saturating CO_2

The activity of Rubisco in higher plants slowly declines over time in the presence of saturating RuBP and CO_2 at 25°C *in vitro*, a phenomenon described as 'fallover'. This decline is due to the binding of RuBP analogues to Rubisco's carbamaylated catalytic sites (Edmondson *et al*. 1990*b*; Zhu and Jensen 1991*a*) and is not due to a decarbamylation of the catalytic site (Edmondson *et al*. 1990*c*; Zhu and Jensen 1991*a*). Pearce and Andrews (2003) demonstrated that the kinetics of 'fallover' can be altered by a mutational loosening of Rubisco's catalytic site on loop 6, causing a decreased rate and extent of fallover. However, this mutational loosening also increased the rate of inhibitor production. Zhu *et al*. (1998) found that increasing lengths of the *C*-terminus, which covers loop 6 during catalysis, improves Rubisco's catalytic performance at higher temperatures. We found that elevated temperatures also reduce the rate and extent of fallover (Fig. 2; Table 1) while increasing XuBP production (Fig. 5). This suggests a temperature-dependent loosening of Rubisco's catalytic site similar to the mutational loosening found by Pearce and Andrews (2003).

The release of inhibitors from Rubisco's catalytic site is described by a two-step process (Pierce *et al*. 1980; Duff *et al*. 2000). The first step is a slow transition from a tightly bound inhibitor–enzyme complex to a more

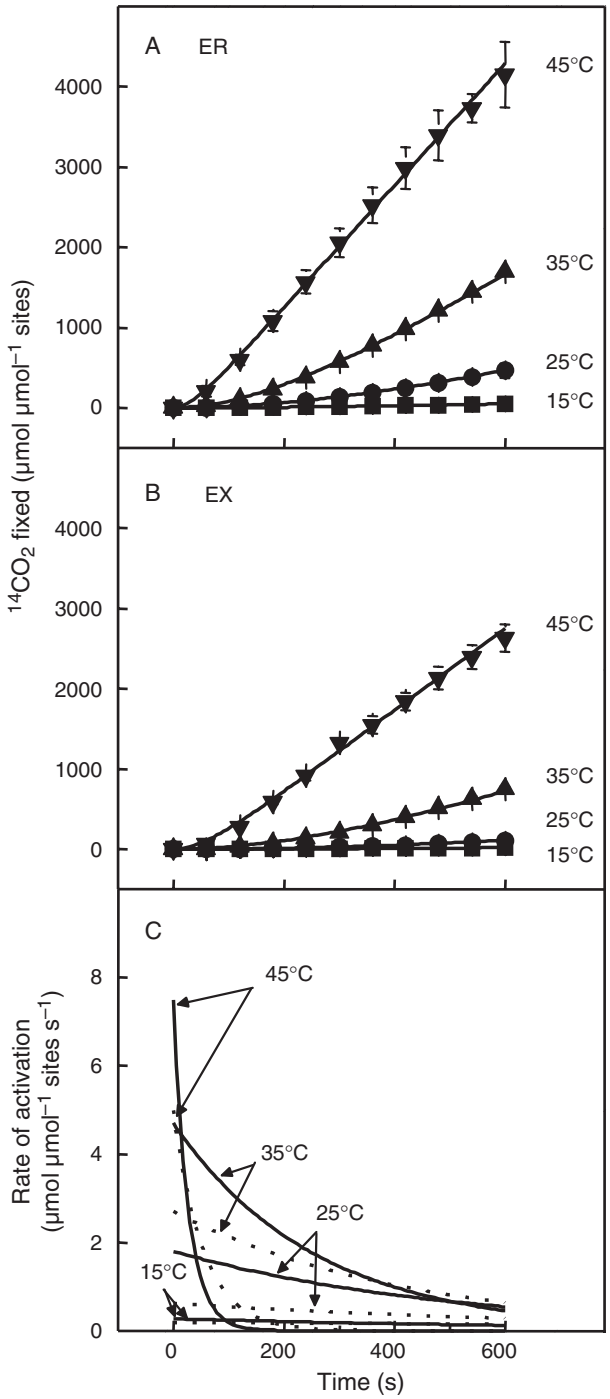

Fig. 4. Activation of Rubisco complexed with RuBP (*A*) and XuBP (*B*) *in vitro* under saturating CO_2 at different temperatures with their corresponding rates of activation (C). Acid-stable product accumulation of uncarbamylated, Mg^{2+} free Rubisco complexed with RuBP and XuBP at 15 (■), 25 (●), 35 (▲) and 45°C (▼). Reaction rates were derived by fitting acid-stable product accumulation data to Eqn 3 (fitted lines in panels *A* and *B*), estimating V_f, and k_{obs}, and applying these estimates to plot the reaction rate *v.* time (*C*) using Eqn 2 while holding $V_i = 0$. Assays were initiated by the addition of the ligand-complexed Rubisco (final concentration of 10 µg mL^{-1}) to an otherwise complete reaction mixture as in Fig. 2 except with 0.75 mM RuBP. $n = 3$.

Table 2. Kinetic parameters for uncarbamylated, Mg^{2+}-free Rubisco taken from Fig. 4

Parameters were estimated using Eqn 1 on the mean of three independent replications of each temperature. $n = 3 \pm$ s.d.

Temperature (°C)	V_f (s^{-1})	k_{obs} (s^{-1})	$t_{1/2}$ (s)
Uncarbamylated, Mg^{2+}-free Rubisco complexed with RuBP (ER)			
15	0.27 ± 0.03	0.0013 ± 0.0003	533
25	1.8 ± 0.32	0.002 ± 0.0002	347
35	4.7 ± 0.43	0.0039 ± 0.0008	178
45	7.5 ± 0.66	0.0368 ± 0.0155	19
Uncarbamylated, Mg^{2+}-free Rubisco complexed with XuBP (EX)			
15	0.19 ± 0.06	0.0004 ± 0.0001	1733
25	0.62 ± 0.22	0.0013 ± 0.0003	533
35	2.7 ± 0.5	0.0024 ± 0.0006	289
45	5 ± 0.36	0.0185 ± 0.0016	37

loosely bound intermediate complex. The slow transition is associated with the opening and closing of loop 6 and the movement of a *C*-terminal strand and an *N*-terminal domain, which surround the catalytic site. The second step is a faster dissociation of the loosely bound inhibitor from the enzyme. It is assumed that the movement of loop 6 is much slower than the dissociation of the inhibitor from the enzyme, and thus the overall rates of binding and

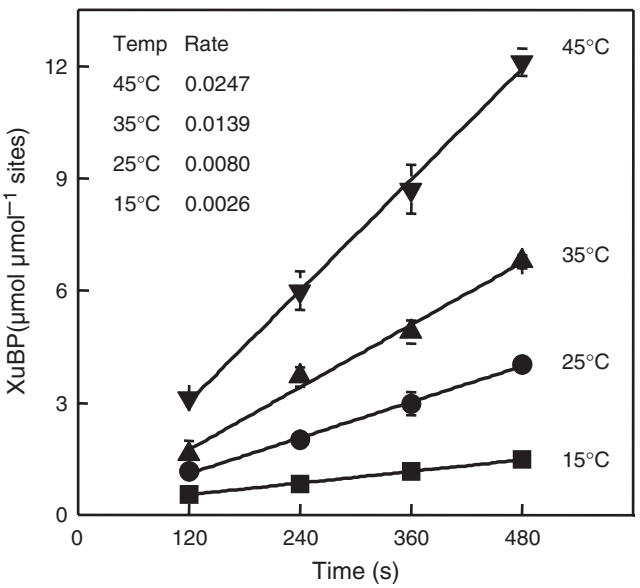

Fig. 5. XuBP production by Rubisco *in vitro* at 15 (■), 25 (●), 35 (▲) and 45°C (▼). Assays was conducted in N_2-sparged buffer containing a final concentration of 3.3 mM RuBP and 6.6 mg mL^{-1} fully activated Rubisco, 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA and stopped with perchloric acid, 1% final concentration. XuBP concentration in the neutralised supernatant was quantified as described in Materials and methods. The inset table contains the calculated rates of XuBP formation at the different temperatures in µmol XuBP µmol sites^{-1} s^{-1}. Rubisco sites were estimated at 1 mg protein = 15 nmol sites. $n = 3$.

Table 3. Rubisco activity

In vitro Rubisco activity at 15, 25, 35 and 45°C after incubating 7.7 mM RuBP in the presence of 2 mg mL^{-1} Rubisco under either 20 mM $NaHCO_3$, N_2-sparged, or O_2-sparged buffer containing 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, and 10 mM borate. Incubation reactions were stopped with perchloric acid, neutralised with K_2CO_3, and the supernatant was assayed in 15 mM $NaHCO_3$ and 0.7 mM fresh RuBP with fresh rubisco, 2.5 μg mL^{-1} final concentration, in 100 mM EPPS–NaOH pH 8.0, 20 mM $MgCl_2$, and 1 mM EDTA. Rubisco and RuBP controls lacked Rubisco or RuBP during the incubation and were assayed equivalently. $n = 3 \pm$ s.d.

Buffer temperature	Rubisco activity (mol CO_2 mol^{-1} Rubisco s^{-1})				
(°C)	20 mM $NaHCO_3$	N_2-sparged	O_2-sparged	Rubisco control	RuBP control
15	0.44 ± 0.01	0.19 ± 0.01	0.175 ± 0.001	0.705 ± 0.002	0.76 ± 0.07
25	1.39 ± 0.01	0.94 ± 0.01	0.56 ± 0.02	2.17 ± 0.002	2.05 ± 0.05
35	3.49 ± 0.01	3.23 ± 0.06	1.15 ± 0.03	4.26 ± 0.03	3.95 ± 0.01
45	6.35 ± 0.02	7.1 ± 0.1	3.47 ± 0.07	7.96 ± 0.09	7.7 ± 0.1

release are determined by the flexible domains around the catalytic site.

Unlike the single amino acid perturbation of loop 6 in the Val335 mutant described by Whitney *et al*. (1999) and Pearce and Andrews (2003), increasing temperature is likely to weaken the interactions between all the flexible domains and the bound inhibitor. This difference in the loosening of the catalytic site is most apparent with the inhibition caused by the oxygenase inhibitors. The Val335 mutant Rubisco was more susceptible than wild type to the oxygenase inhibitors with the inhibition going to completion (i.e. $V_f = 0$; Pearce and Andrews 2003). However, the inhibition by these same ligands decreased as temperature increased (Table 3). This suggests that the fundamental character of the catalytic site does not change with temperature as it has been in the Val335 mutant.

The fallover reactions of our experiments were measured over an extended period of 60 min. Kane *et al*. (1998) found that RuBP can spontaneously oxidise to form PdBP in stock preparations, and during the long assay times of fallover experiments. This suggests that fallover assays at elevated temperatures may be confounded by a greater production of the tight-binding inhibitors PdBP and CtBP at higher temperatures. We found no effect of temperature on the rate of fallover with RuBP that had been incubated in the absence of Rubisco at the temperatures studied (Table 1). However, the extent of fallover was greater in all cases. Apparently, under the mild conditions used in our experiments the spontaneous oxidation of RuBP was consistent among all four temperatures.

Rate of inhibitor release from Rubisco's uncarbamylated catalytic site increases with temperature

Consistent with the hypothesis that temperature loosens the catalytic site, the rate of activation (k_{obs}) of metal-free, uncarbamylated Rubisco complexed with RuBP (Fig. 4*A*; Table 2) or XuBP (Fig. 4*B*; Table 2) increased with temperature. Because we measured the final carboxylation rate of Rubisco, the overall activation process involves the initial release of the inhibitor from the catalytic site, the carbamylation of the catalytic site, and finally the binding and carboxylation of RuBP. Heat should increase the rate of all of these reactions simultaneously, though possibly not equivalently. It is assumed that the rates of carbamylation and carboxylation are rapid and equivalent between the RuBP- and XuBP-complexed enzymes at the same temperatures. Therefore, the limiting reaction in the activation of Rubisco should be the dissociation of the ligand from the catalytic site. While increasing temperature increased the rate of activation for both ligands, the rate of activation for the RuBP-complexed enzyme increased more than the XuBP-complexed enzyme. This is consistent with a tighter binding of XuBP to the catalytic site than RuBP (Zhu and Jensen 1991*b*). The activation rate determined at 15°C is not well resolved because the estimated half-time is roughly three times longer than the length of the experiment. However, the estimates at 25°C, and particularly 35 and 45°C demonstrate an increasing rate of dissociation of both ligands from the catalytic site.

While V_f for the RuBP-complexed enzyme reached full activity as compared to fully activated Rubisco V_i values in Table 1, V_f for the XuBP-complexed enzyme did not (Fig. 4*B*; Table 2). Either a fraction of the XuBP complexed catalytic sites did not dissociate or carryover XuBP acted as a competitive inhibitor of RuBP carboxylation during catalysis. XuBP is known to bind more tightly to the uncarbamylated catalytic site of Rubisco (Zhu and Jensen 1991*b*), but inhibition of catalytic activity at 25°C under alkaline pH is not due to decarbamylation of the enzyme (Edmondson *et al*. 1990*c*), but to the binding of inhibitors to the carbamylated catalytic site. Pearce and Andrews (2003) reported at 25°C approximately a 60% inhibition of fully carbamylated Rubisco with XuBP concentrations near 2.6 μM, which was the carryover concentration in our experiments. This provides a plausible explanation for the 53% difference in final Rubisco activities between RuBP- and XuBP-complexed enzymes in our experiments. This

suggests that competitive inhibition and not tight binding of the uncarbamylated catalytic site is responsible for the discrepancy between final activities in RuBP- and XuBP-complexed Rubiscos.

Inhibitor production at Rubisco's catalytic site increases with temperature

Salvucci and Crafts-Brandner (2004) recently demonstrated a temperature-dependent increase in XuBP production by Rubisco under a N_2 atmosphere, which was confirmed here (Fig. 4). Although our experiments confirmed the temperature-dependent increase in XuBP, they were inconclusive for the tight-binding inhibitors (data not shown). While Kim and Portis (2004) demonstrated a temperature-dependent increase in H_2O_2 production by Rubisco, it was unclear whether this resulted in an increase of both PdBP and CtBP or just PdBP. Pearce and Andrews (2003) reported a conversion of PdBP into CtBP by the Val335 mutant. This suggests that if temperature loosens the catalytic site similar to the Val335 mutant, it may also facilitate the conversion of PdBP into CtBP as the Val335 mutant does.

Implications for in vivo *function of Rubisco*

Although Rubisco can be fully carbamylated and catalytically competent *in vitro*, Rubisco's helper enzyme, Rubisco activase, has been shown to be essential for full Rubisco carbamylation and function *in vivo* (Salvucci *et al*. 1985; Mate *et al*. 1993, 1996; Eckardt *et al*. 1997). Rubisco activase aids in the release of sugar phosphates from Rubisco catalytic sites *in vitro* (Robinson and Portis 1988; Portis 1992; Wang *et al*. 1992; Wang and Portis 1992). The precise mechanism by which activase promotes carbamylation *in vivo* remains unclear, but it has been suggested that activase functions to open closed loop structures at the catalytic sites thus releasing bound inhibitors and sugar phosphates (Portis 2003). Our *in vitro* studies suggest a loosening of the loop structure at Rubisco's catalytic sites at higher temperature for both carbamylated and uncarbamylated sites, which could on the one hand suggest a lesser role of Rubisco activase under these conditions. However this is countered by an increased rate of inhibitor production at Rubisco's catalytic site (Fig. 5; Kim and Portis 2004; Salvucci and Crafts-Brandner 2004), and by the observed tendency of activase to denature at high temperatures (Eckardt and Portis 1997; Salvucci *et al*. 2001).

Pearce and Andrews (2003) pointed out that there are many examples of algal and bacterial Rubiscos that lack fallover kinetics *in vitro* and that this lack of fallover is generally associated with a lower CO_2 / O_2 specificity as well as a lower catalytic effectiveness (k_{cat} / K_m for carboxylation). They suggested that the attainment of greater carboxylase specificity has come at a cost of making the closure of loop 6 over the substrate so precise that substrate analogues cannot escape the Rubisco site without the assistance of Rubisco activase. Specificity (but not catalytic effectiveness) declines with increasing temperatures in Rubiscos from C_3 species (Badger and Collatz 1977; Jordan and Ogren 1984; Galmes *et al*. 2005). Our results show that the temperature-dependent decline in Rubisco specificity also correlates with a decline in fallover.

Salvucci and Crafts Brandner (2004) reported variation in Rubisco activase properties in species from diverse thermal habitats. Sage (2002) reported higher catalytic turnover rates for Rubisco carboxylation of C_3 species from cool climates. It may also be of interest to examine the Rubisco specificity and fallover kinetic in species from diverse thermal habitats.

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